Morphological and morphometric characteristics of the epididymis in the Neotropical bats *Eumops glaucinus* and *Molossus molossus* (Chiroptera: Molossidae)

Mateus R. Beguelini[1], Bruno F. S. Sergio[2], Fábio L. J. Leme[2], Sebastião R. Taboga[1], Eliana Morielle-Versute[2*]

1 Department of Biology, São Paulo State University – UNESP/IBILCE, São José do Rio Preto, São Paulo, Brazil 15054-000
2 Department of Zoology and Botany, São Paulo State University – UNESP/IBILCE, São José do Rio Preto, São Paulo, Brazil 15054-000

*Autor correspondente: morielle@ibilce.unesp.br

ABSTRACT
The ability of certain species of bats to store viable spermatozoa in the cauda epididymis, for periods of many months beyond the end of spermatogenesis was first recognized over a century ago. However, information about the bat epididymis is still scarce or absent. Thus, this study aimed to characterize and to compare morphologically and morphometrically the regional histology of the epididymis of *Eumops glaucinus* and *Molossus molossus* (Chiroptera: Molossidae). Histologically, the epididymis of both species was subdivided into 4 segments: initial segment, caput, corpus and cauda. In comparing the two species, it was observed that the tubular and luminal diameters and percentage of interstitial tissue showed significant differences in all segments. The epithelial height, in both, is greater in the initial segment with a decrease until the cauda epididymis. In relation to the luminal diameter, both species showed a gradual increase from the initial segment to the cauda. The percentage of epithelium, lumen and interstitial tissue varied between both, sometimes *M. molossus* showing a significantly higher percentage, and other times, *E. glaucinus*. In both species, the principal cell was the most abundant (> 77%), followed by basal cells at approximately 13% and apical cells at 4% in all segments. Spermatozoa were observed in greater amounts in corpus and cauda epididymis segments. In summary, ours results show that, despite that the species analyzed belong to different genera and have different breeding cycles, the epididymis exhibits similarities in the two species and morphometric and composition differences compared to the majority of mammals.
Keywords: Epididymis; Molossidae; Chiroptera

INTRODUCTION

The epididymis is a highly convoluted tubule which connects the testis to the ductus deferens and is an important segment of the excurrent duct system of the testes that performs a variety of functions (Beu et al. 2009). The epididymal duct is not a mere sperm-transporting duct; it is an androgen-dependent organ, which plays a key role in the transport, maturation and storage of spermatozoa. The epididymal epithelium is responsible for creating the ideal conditions that make spermatozoa fertile and motile via absorption, secretion, synthesis, and metabolic activity (Aguilera-Merlo et al. 2005; Ahmed et al. 2009; Beu et al. 2009).

Studies on the mammalian epididymis (Ramos and Dym 1977; Hart and Schoning 1984; Oke et al. 1988; Goyal and Williams 1991; Bedford 1994; Calvo et al. 1999; Crichton and Krutzsch 2000; Schimming and Vicentini 2001; Schimming et al. 2002; Aguilera-Merlo et al. 2005; Domeniconi et al. 2007) have shown that this organ can be divided into distinct regions according to the biochemical, morphological and morphometric characteristics of its segments. Various divisions have been proposed and the most widely used is that dividing the organ into the initial segment, caput, corpus and cauda epididymis (Serre and Robaire 1998).

The ability of certain species of bats to store viable spermatozoa in the cauda epididymis, for a long time (6 - 7 months), beyond the interruption of spermatogenesis, was first recognized over a century ago (Wimsatt 1969; Hosken et al. 1996; Crichton and Krutzsch 2000). However, information about the bat epididymis is still scarce or absent, mainly in Neotropical species.

Eumops glaucinus and *Molossus molossus* are exclusively Neotropical species that belong to the family Molossidae, which is widely distributed around the globe in temperate and tropical regions. In spite of the large number of species (86 species grouped in 16 genera) (Simmons 2005), it is one of the most poorly studied families of bats especially due to the behavior the animals, with high and fast flights, which has hampered studies of this family.

The majority of the bats from the Neotropics show seasonal reproductive patterns, intimately linked to abiotic factors, such as temperature, rainfall and availability of food (Fleming et al. 1972; Krutzsch 2000). Monoestrous species of the family Molossidae have birth associated with spring, while some species of the genera *Eumops* and *Molossus* are polyestrous, showing a postpartum estrus (Carter 1970; Fabián and Marques 1989).

Eumops glaucinus is a polyestrous species that shows great geographic variation in their reproductive patterns (Belwood et al. 1992; Best et al. 1997). This species has only one annual breeding cycle in Florida - USA (pregnant females from August to September), but there is evidence of reproductive activity throughout most of the year in Cuba (Best et al. 1997). On the other hand, studies in *M. molossus* indicated that, in Brazil, this species shows two annual reproductive breeding seasons, one in March-April and another in November, and

sexually active males were found along almost the entire year (Fabián and Marques 1989).

Thus, the present study aims to extend the knowledge about the reproduction of *Eumops glaucinus* and *Molossus molossus* characterizing and comparing morphologically and morphometrically the regional histology of the epididymis of the specimens of these species, in order to provide a detailed structural baseline for specific studies, such as the functional role of different epididymal regions and cells in sperm maturation and storage.

MATERIAL AND METHODS

Animals, tissue collection and processing

Five sexually mature specimens of *Eumops glaucinus* (Wagner 1843) and *Molossus molossus* (Pallas 1766) were analyzed. The specimens are in the Chiroptera Collection at São Paulo State University (DZSJRP-UNESP). The animals were collected in the northwest part of São Paulo State, Brazil (49W22'45" 20S49'11") between July and November 2008. This period shows a dry winter and early spring and is the coldest season of the year (15–25ºC). The experimental procedures were approved by the Ethics Committee of São Paulo State University (UNESP) (Process: 013/09 – CEEA), and the capture and captivity of bats was authorized by the Brazilian institution responsible for wild animal care (Instituto Brasileiro do Meio Ambiente, IBAMA – Process: 02027.001957/2006-02).

After dead by cervical dislocation the animals were accommodated in the supine position on the board of dissection. An incision was made in the abdominal region in the caudal direction and the skin hit to the sides. Once exposed and removed, the testis and epididymis were photographed for gross documentation with a camera HP Photosmart M627 (6.0 mm - 18.0 mm), and then they were immersed in Bouin fixative solution for at least 24 hours, dehydrated in graded series of ethanol, embedded in glycol methacrylate (Historesin, Leica Instruments), and sectioned (1 μm thickness) using a Leica RM 2155 microtome. Tissue sections obtained from serial sections of epididymis were stained with hematoxylin-eosin (Ribeiro and Lima 2000).

The reproductive status of each specimen was histologically evaluated with the aim to confirm its sexual maturity.

Morphological and morphometric analysis of epididymis

The following measurements were performed in epididymis: tubular and luminal diameters, epithelial height (excluding the stereocilia), abundance of each cell type and the relative percentage of tissue (epithelium, lumen and interstitium).

The tubular and luminal diameters and the epithelial height were determined using Axiovision 3.1 for Windows® software for image analysis. The analysis was performed in 200 epididymal tubule cross-sections per animal at 400x magnification for each epididymal segment (at least ten transverse epididymal sections were analyzed). Only transverse epididymal sections were included in this study. The epithelial height was taken as the linear length of the principal cells, from the base of the epithelium (basal lamina) to the apical edge (excluding the stereocilia), the luminal diameter as the longest measurement from one apical edge to the other, and the tubular diameter as the longest distance between basal-basal laminas.

The abundance of cell types was analyzed for each segment in the same 200 epididymal tubule cross-sections per animal at 400x magnification and expressed as percentage of the total population. Abundance was estimated by counting nuclei.

The relative percentages of epithelium, lumen and interstitial tissue were estimated using the Image-Pro-Plus software, version 6.0 (© Media Cybernetics) for Windows®. The analyses of the tissue segments were carried out according to the procedure of Weibel using a 168-point grid test system (Weibel 1963). The data were obtained from 30 random microscopic fields per segment at 200x magnification. The relative percentage (%) was calculated after counting the number of points that coincided with each of the tissue compartments (epithelium, lumen of ducts and interstitial tissue).

RESULTS

In both species the testes are migratory ie, they were observed located outside the abdominal cavity, between the skin and muscles near the crest of the pubis, laterally to the base of the penis (parapenial), or a testis located outside and another inside the abdominal cavity or even both testes located within the abdominal cavity, dorsally arranged below the kidneys. All the testes were considered functionally active because numerous spermatids read for release were observed inside the seminiferous tubules (Fig. 1).

The epididymis of the two species of bats analyzed corresponds to a single elongated and convoluted tubule that lies fully and firmly connected laterally to the testis. Macroscopically, the four main segments, initial (Is), caput (Ca), corpus (Cp) and cauda (Cau), were observed (Fig. 2). Histological analysis showed that in both species the initial segment is located between the confluence of the tubules from the rete testis and the caput epididymis (Fig. 3A-B). The epididymal corpus represents the medial segment of the epididymis (Fig. 3C) and the cauda epididymis, the end segment of the epididymis, which communicates directly with the ductus deferens (Fig. 3D).

In both *E. glaucinus* and *M. molossus*, the entire epididymal duct is covered by a myo-connective tissue sheath and is composed of a pseudostratified columnar epithelium with extensive stereocilia (Fig. 4). The epithelium is composed mainly of three cell types: the principal, basal, and apical cells. However, two other cell types are observed, halo and clear cells. The principal cells are the most predominant type, they are columnar cells, which extend from the basal lamina to the lumen, and show a highly secretory apex, which displaces the round-ovoid nucleus to the middle or parabasal region of the cell. The basal cell is firmly apposed to the basal lamina, does not reach the tubular lumen, and has an elongated nucleus. The apical cell is also a columnar cell

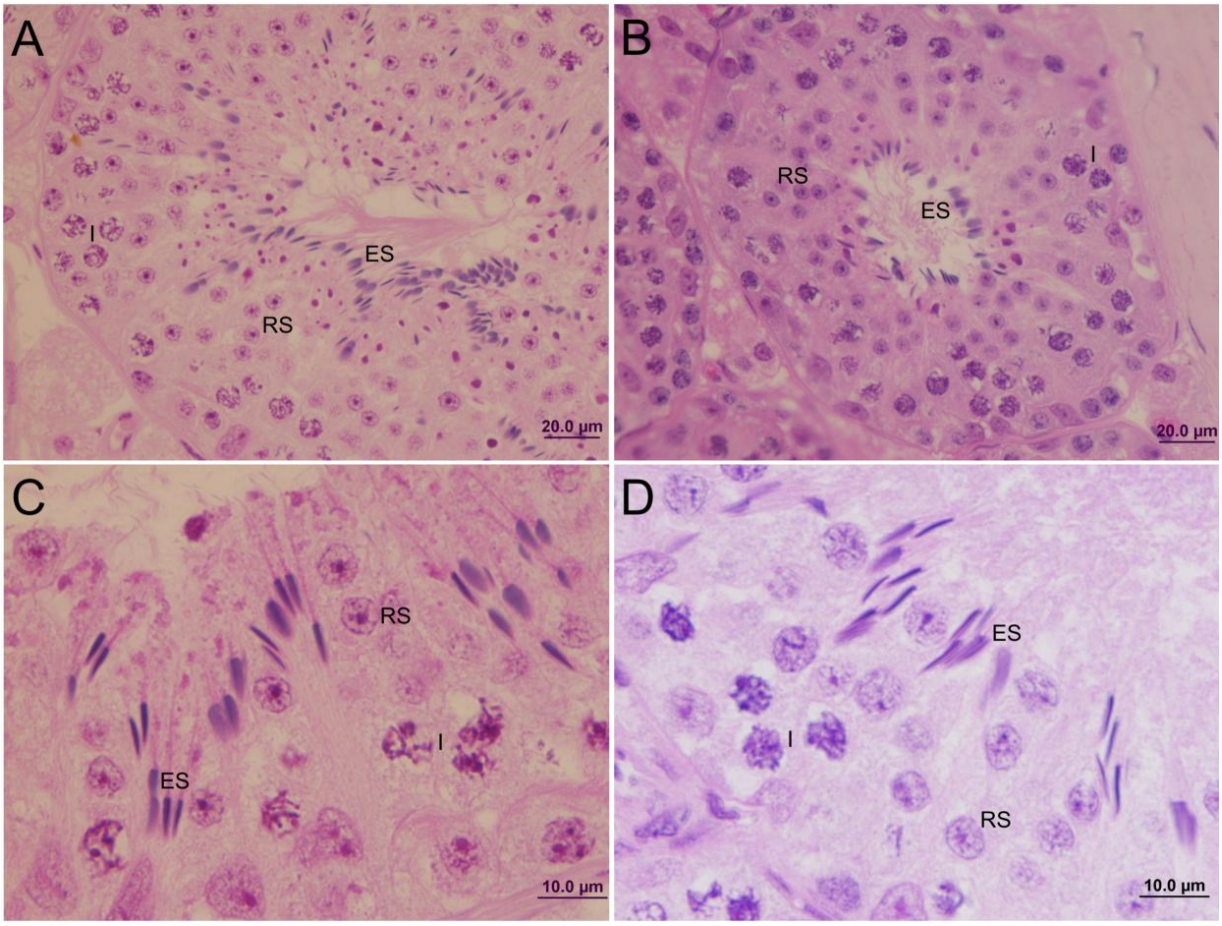

Fig. 1. General arrangement of the testicular epithelium stained with hematoxylin-eosin of *Eumops glaucinus* (A and C) and *Molossus molossus* (B and D). Note primary spermatocytes (I), round spermatids (RS), and elongated spermatids (ES) ready for release.

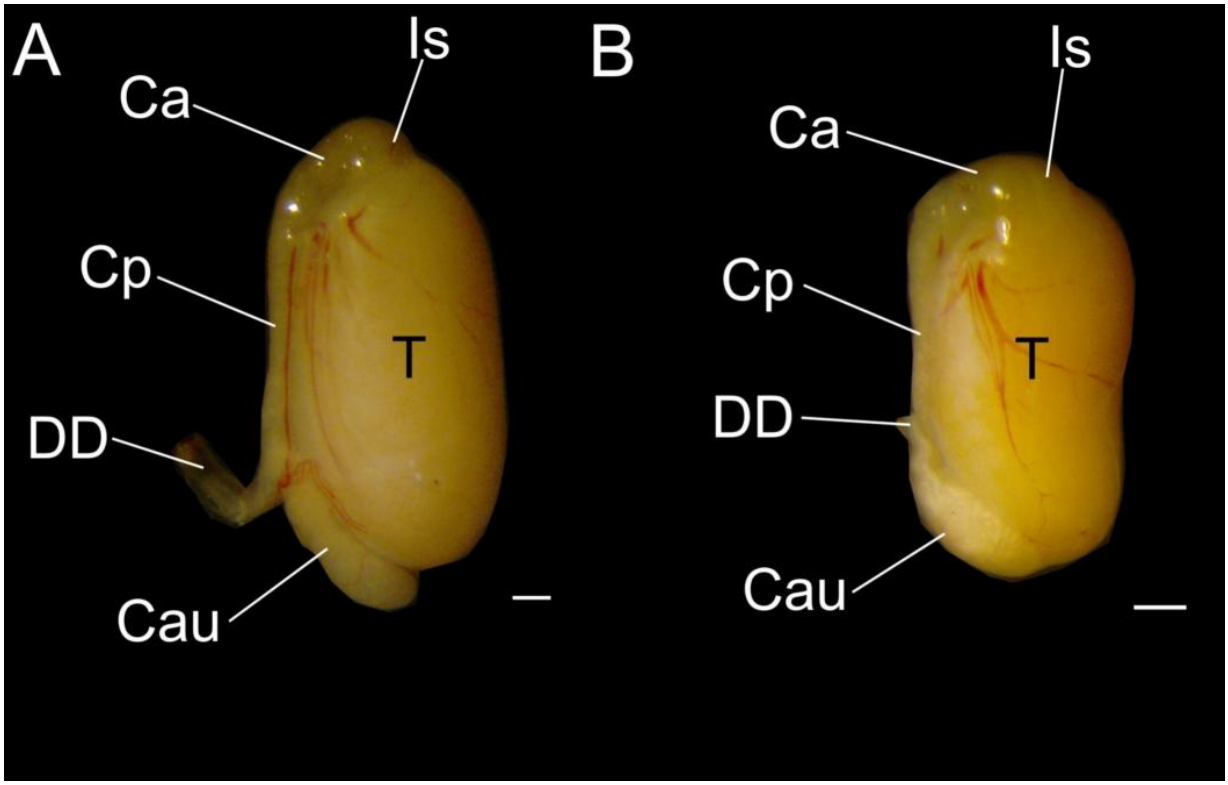

Fig. 2. Gross anatomy of the testes and epididymis of *Eumops glaucinus* (A) and *Molossus molossus* (B). (Ca, caput; Cau, cauda; Cp, corpus; DD, ductus deferens; Is, Initial segment; T, testis). Scale bars: 1 mm.

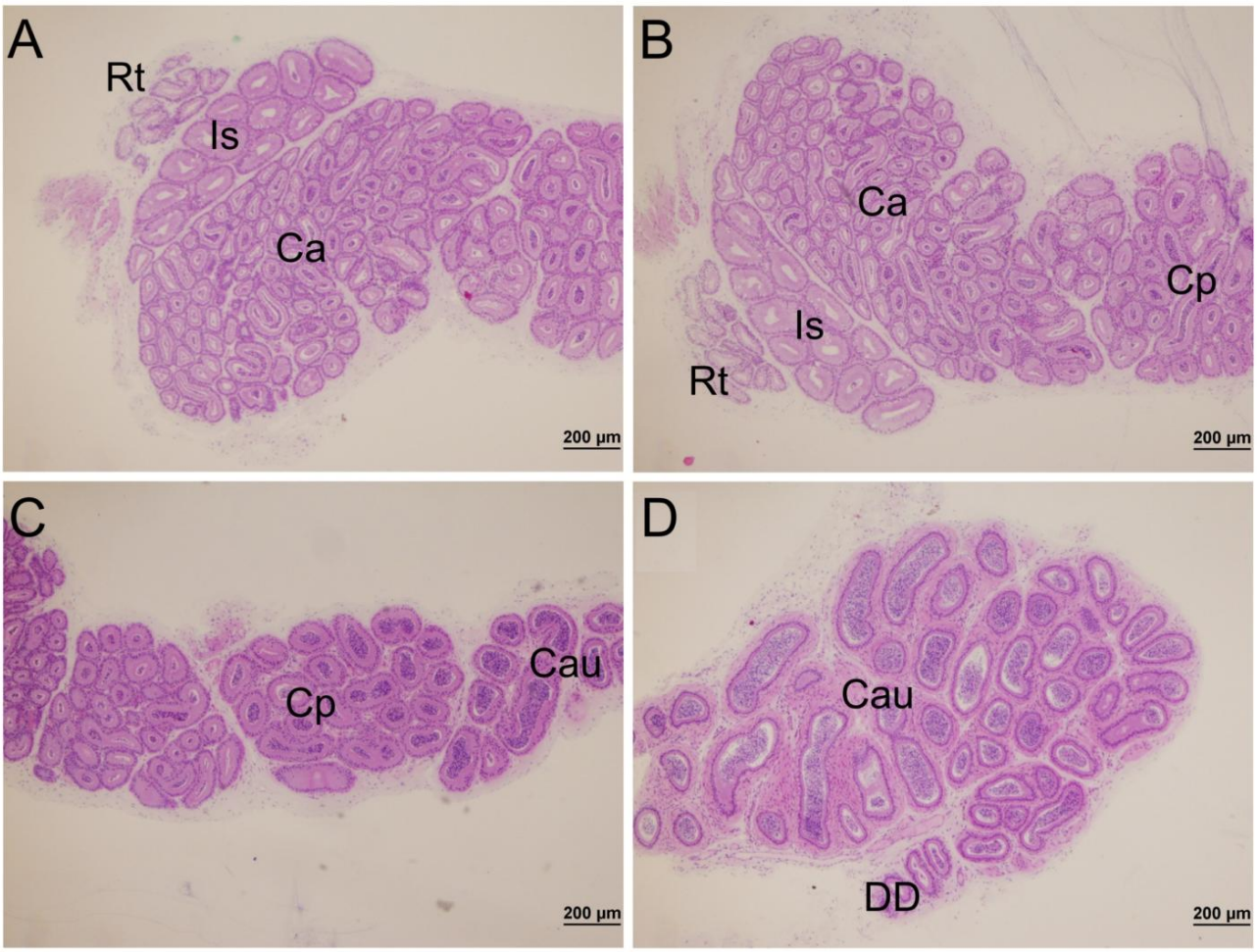

Fig. 3. General arrangement of the epididymis of *Molossus molossus* stained with hematoxylin-eosin. A-B. Initial region of the epididymis. C. Middle region of the epididymis. D. End region of the epididymis. (Ca, caput; Cau, cauda; Cp, corpus; DD, ductus deferens; Is, Initial segment; Rt, rete testis).

that extends from the basal lamina to the lumen; however, it has a slender shape in the basal and parabasal region, with a wide cytoplasmic apical region that shelters the nucleus and touches the lumen (Fig. 4).

The halo and clear cells are observed in relatively low percentages and in only some segments. Therefore, they are not considered in the total cell number. In *M. molossus*, few halo and clear cells are seen in the epididymal corpus (Fig. 5A-B), and in *E. glaucinus*, clear cells are visible in the initial and caput segment of epididymis (Fig. 5C). In *E. glaucinus*, a few cells are also seen showing morphology similar to principal cells, but with a large elongated nucleus (Fig. 5D).

The four regions of the epididymis showed regional differences in the measures of epithelial height, tubular and luminal diameters, relative percentage of tissues and relative cell composition, which are summarized in Table 1 and Figure 6.

The initial segment is composed, in both species, of an irregular and tall epithelium which is the greatest epididymal epithelial height observed (Fig. 6A-D); on the other hand, this segment is the shorter segment of the epididymis, showing few tubules and differences in all measurements between the species analyzed. The tubular (TD) and luminal (LD) diameters and the epithelial height (EH) in *E. glaucinus* (TD: 121.4 ± 16 µm; LD: 34.9 ± 10.9 µm; EH: 41.6 ± 4.7 µm) are significantly higher than in *M. molossus* (TD: 89.7 ± 22.9 µm; LD: 26.4 ± 8.8 µm; EH: 32.8 ± 11.3 µm).

Inversely, the relative percentage of epithelium (E) and lumen (L) of *E. glaucinus* (E: 59.5 ± 7.8%; L: 7.6 ± 3.5%) is statistically lower compared to *M. molossus* (E: 68.8 ± 4.7%; L: 10.9 ± 3.5%). However, the interstitium (It) shows a significantly higher proportion (32.9 ± 8.6%) in *E. glaucinus* than in *M. molossus* (20.3 ± 5.4%) (Fig. 6A and B).

The epithelium of the initial segment contains many vacuoles and all the cell types described, with a great abundance of principal cells (82.2 ± 6.6% in *E. glaucinus* and 77.1 ± 3.6% in *M. molossus*) and some basal cells located directly over the basal lamina (11.8 ± 4.6% and 14.4 ± 3.1%, respectively) (Fig. 6E and F). The apical cells represent 5.9 ± 4.6% in *E. glaucinus* and 8.4 ± 3.2% in *M. molossus*. The proportion of each cell type is significantly different between the two species. The lumen is irregular in both species, represents the smaller percentage of the tissue, and is devoid of spermatozoa.

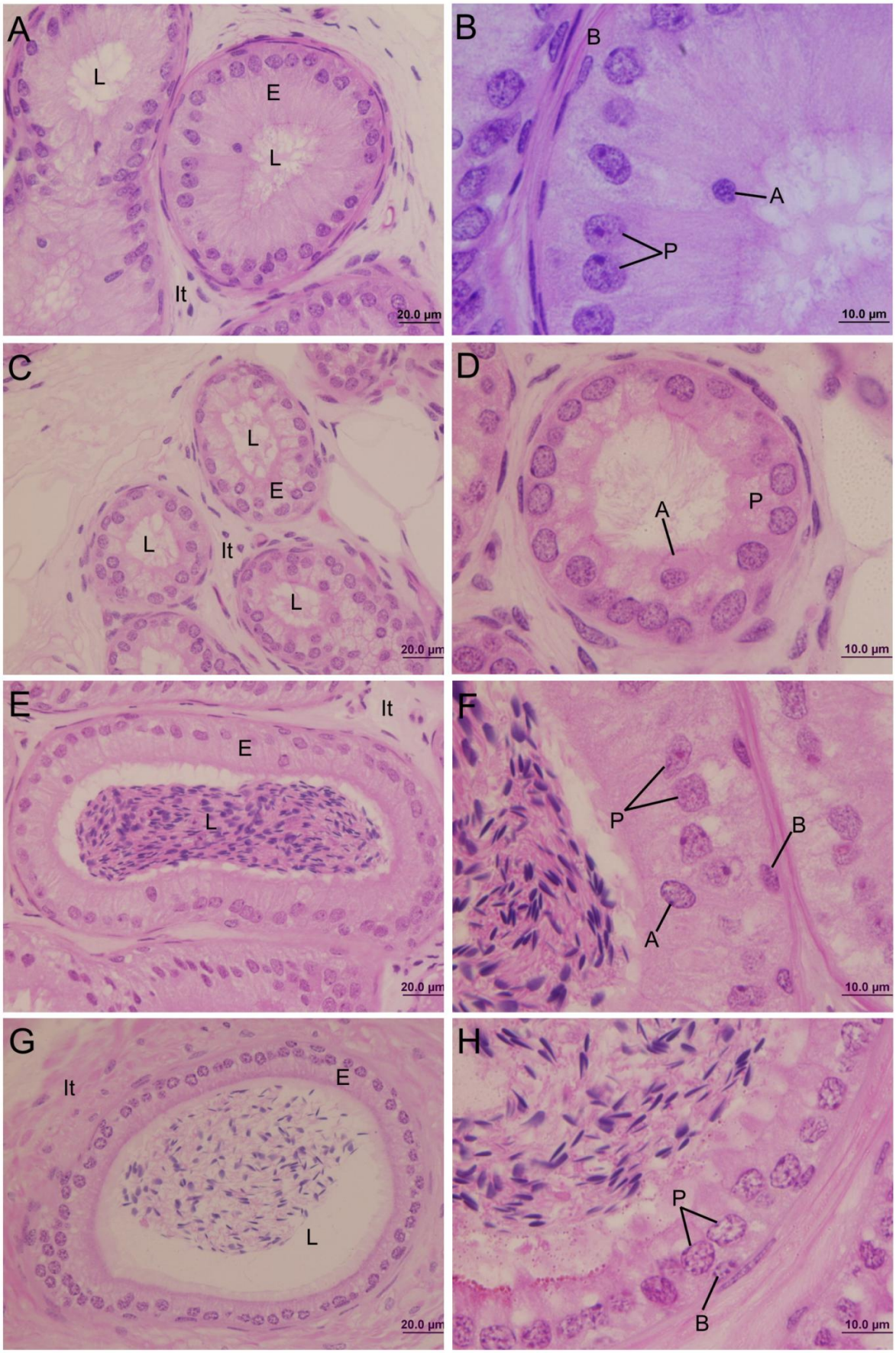

Fig. 4. General arrangement of the epididymis of *Eumops glaucinus* stained with hematoxylin-eosin. A-B. Initial segment. C-D. Caput. E-F. Corpus. G-H. Cauda. (A, apical cell; B, basal cell; E, epithelium; It, interstitium; L, lumen; P, principal cell).

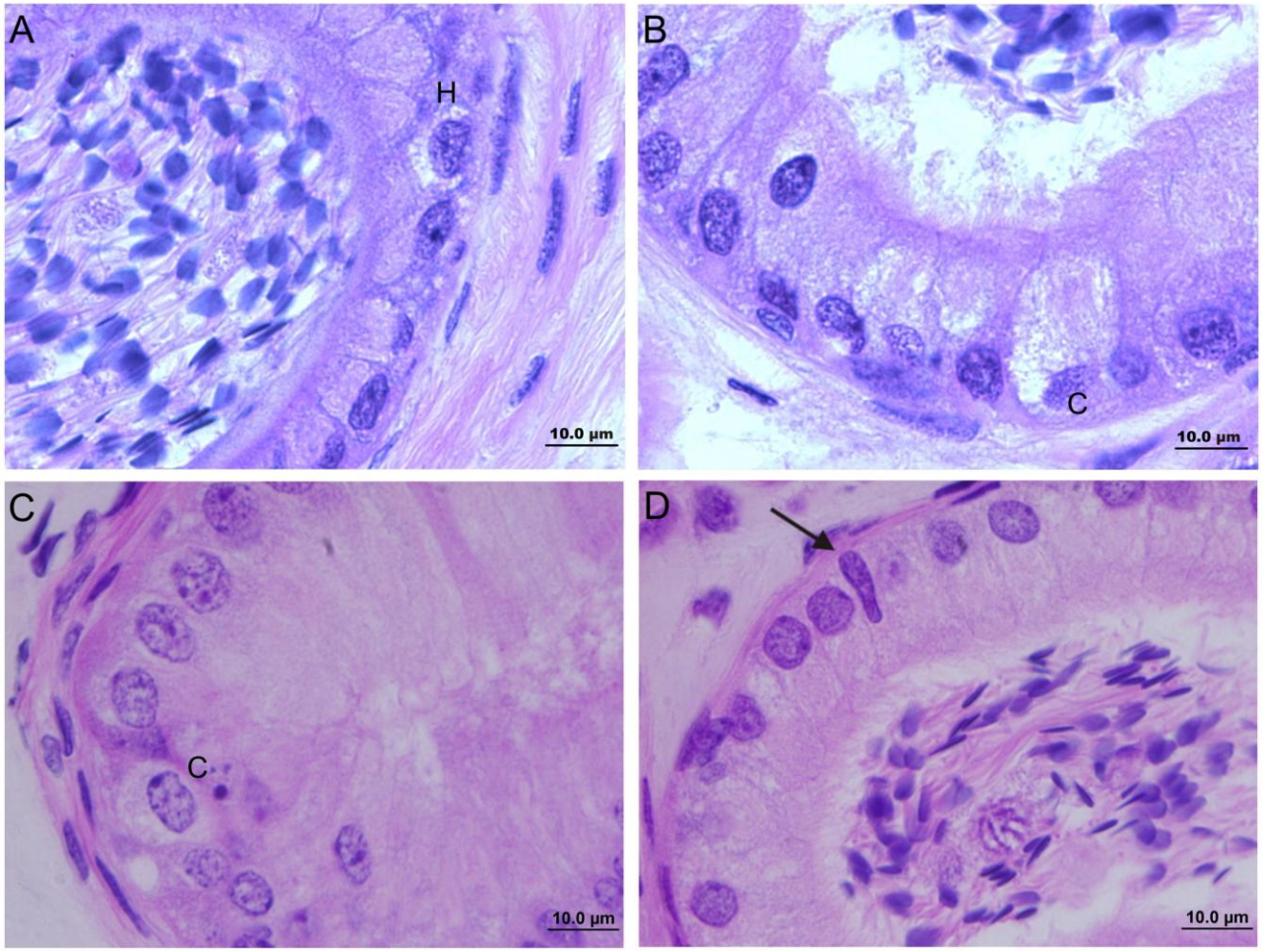

Fig. 5. General arrangement of the epididymis of *Molossus molossus* (A-B) and *Eumops glaucinus* (C-D) stained with hematoxylin-eosin. A. Note the halo cell (H) firmly apposed to the basal lamina, showing a highly stained nucleus with pale cytoplasm. B-C. Note the clear cells (C) with a pale appearance. D. Note the cell with a morphology similar to principal cells, but with a large elongated nucleus (arrow).

The tubular diameter and the epithelial height of the epididymal caput are significantly lower than those of the initial segment in both species (TD: 84.6 ± 6.8 µm; EH: 24.5 ± 3.9 µm in E. glaucinus; TD: 64.9 ± 10.5 µm; EH: 20.2 ± 3.7 µm in M. molossus), and they differ significantly between the two species (Fig. 6C and D). On the other hand, the luminal diameter is not significantly different from that of the initial segment in both species, and statistically different between the two.

The relative percentages of epithelium, lumen and interstitial tissue of the caput epididymis are significantly different from those of the initial segment in *E. glaucinus* (E: 62.1 ± 9.8%; L: 13.2 ± 4.1%; It: 24.7 ± 12.3%) (Fig. 6A and B); however in *M. molossus*, they are not statistically different (E: 70.3 ± 5.7%; L: 12.3 ± 3.9%; It: 17.3 ± 5.2%). The amount of epithelium and interstitial tissue is also statistically different between the two species.

The number of apical cells in the caput epididymis is significantly lower than in the initial segment in both species (*E. glaucinus* = 3.1 ± 2.6% and *M. molossus* = 2.8 ± 2.4%). On the other hand, the number of principal cells is significantly higher in the caput epididymis than in the initial segment in *E. glaucinus* (84.2 ± 5.1%) (Fig. 6E and F).

This segment has tubules with lumen devoid of spermatozoa and others that contain few spermatozoa.

In the corpus epididymis, there is a significant increase in the tubular and luminal diameters and epithelial height in both species (TD: 100.7 ± 12.7µm; LD: 46.4 ± 11.1 µm; EH: 26.1 ± 3.9 µm in *E. glaucinus*; TD: 89.6 ± 14.4 µm; LD: 40.5 ± 14.8 µm; EH: 23.8 ± 5.1 µm in *M. molossus*) (Fig. 6C and D). The difference between the species is also statistically significant.

A significant increase in the percentage of lumen along with a concomitant decrease in amount of epithelium was observed in *M. molossus*, with the percentage of principal cells decreasing statistically in contrast to the increase in basal cell percentage (Fig. 6 B and F). In *E. glaucinus*, a significant increase was noted in the lumen with a decrease in the interstitial tissue, with the number of cells very similar to the preceding segment (Fig 6A and E). In both species, the lumen of this segment contains spermatozoa.

Some interesting features distinguish the cauda epididymis from the other segments. The tubular and luminal diameters increase significantly (TD: 114.8 ±

Table 1. Structural parameters in each epididymal segment of *Eumops glaucinus* (*Eg*) and *Molossus molossus* (*Mm*). Sp: species

Sp	Segment	Tubular Diameter*	Luminal Diameter*	Epithelium Height*	Relative Tissue Percentage**			Relative Cell Composition**		
					Epithelium	Lumen	Interstitium	Apical	Basal	Principal
Eg	Initial	121.4 ± 16	34.9 ± 10.9	41.6 ± 4.7	59.5 ± 7.8	7.6 ± 3.5	32.9 ± 8.6	5.9 ± 4.6	11.8 ± 4.6	82.2 ± 6.6
	Caput	84.6 ± 6.8	33.9 ± 6.8	24.5 ± 3.9	62.1 ± 9.8	13.2 ± 4.1	24.7 ± 12.3	3.1 ± 2.6	12.9 ± 6.4	83.9 ± 5.7
	Corpus	100.7 ± 12.7	46.4 ± 11.1	26.1 ± 3.9	63.3 ± 7.0	22.2 ± 7.4	14.5 ± 5.4	4.6 ± 3.2	13.4 ± 5.1	82.1 ± 6.6
	Cauda	114.8 ± 21.8	78.5 ± 19.5	16.9 ± 3.3	42.5 ± 13.5	32.4 ± 6.5	25.1 ± 13.4	3.4 ± 2.1	12.3 ± 3.9	84.3 ± 3.8
Mm	Initial	89.7 ± 22.9	26.4 ± 8.8	32.8 ± 11.3	68.8 ± 4.7	10.9 ± 3.5	20.3 ± 5.4	8.4 ± 3.2	14.4 ± 3.1	77.1 ± 3.6
	Caput	64.9 ± 10.5	25.5 ± 7.8	20.2 ± 3.7	70.3 ± 5.7	12.3 ± 3.9	17.3 ± 5.2	2.8 ± 2.4	12.9 ± 3.9	84.2 ± 5.1
	Corpus	89.6 ± 14.4	40.5 ± 14.8	23.8 ± 5.1	64.3 ± 8.2	15.4 ± 5.5	20.4 ± 8.5	3.2 ± 4.7	18.3 ± 4.9	78.5 ± 7.0
	Cauda	108.4 ± 12.4	74.7 ± 13.6	16.9 ± 2.9	38.1 ± 13.4	24.1 ± 6.0	47.9 ± 11.9	3.3 ± 3.2	14.2 ± 4.9	82.5 ± 5.9

* Mean ± Standard Deviation in µm. ** Mean ± Standard Deviation of the percentage of total (% of total).

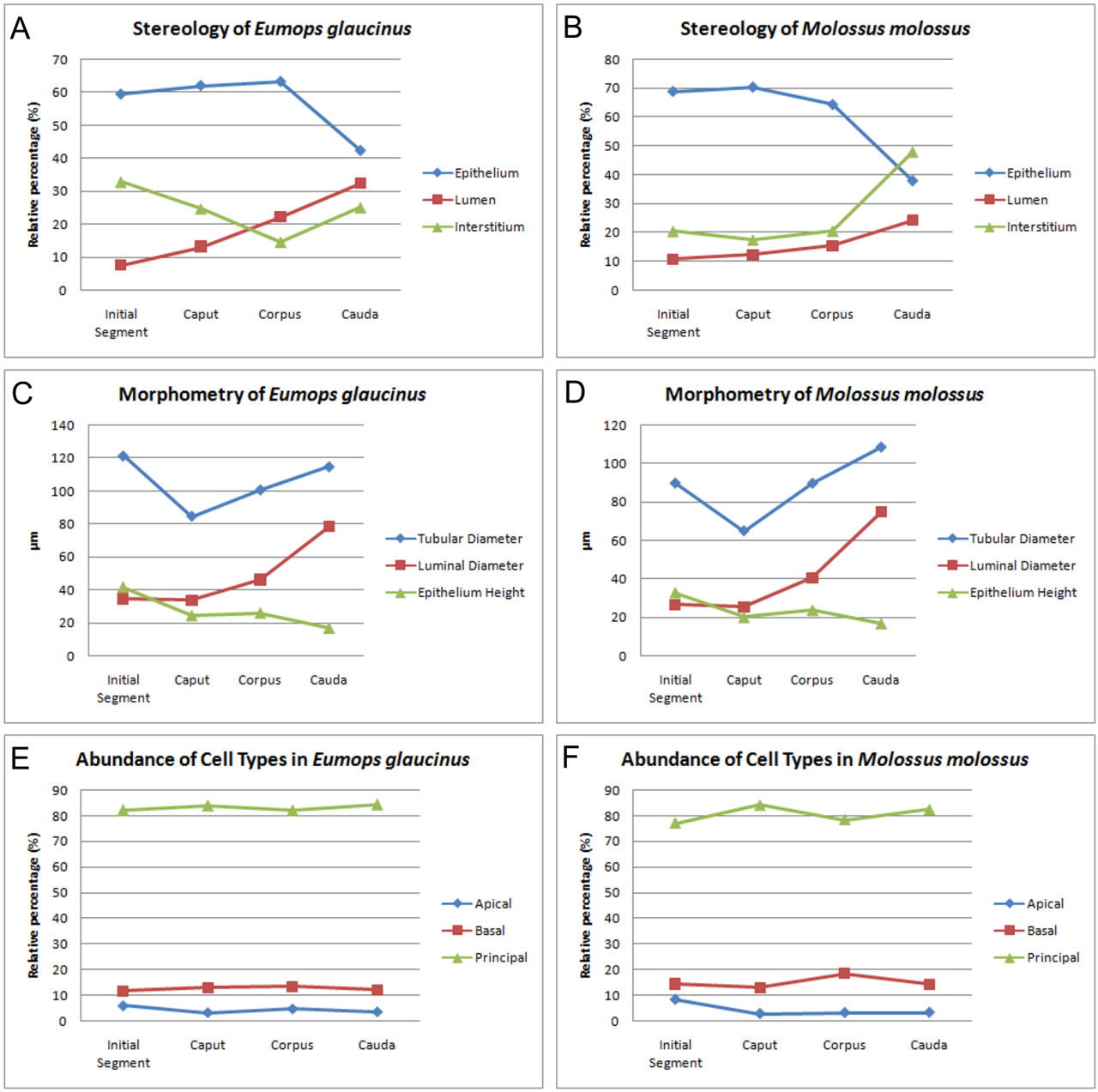

Fig. 6. Graphs showing the morphometric data of *Eumops glaucinus* (A, C and E) and *Molossus molossus* (B, D and F) in each segment of the epididymis. A-B. Relative percentage of epithelium, lumen and interstitial tissue. C-D. Mean values of the tubular and luminal diameter and epithelium height. E-F. Abundance of cell types (apical, basal and principal cells).

21.8 µm; LD: 78.5 ± 19.5 µm in *E. glaucinus*; TD: 108.4 ± 12.4 µm; LD: 74.7 ± 13.6 µm in *M. molossus*), and the epithelium decreases in height (16.9 ± 3.3 µm in *E. glaucinus* and 16.9 ± 2.9 µm in *M. molossus*) in both species (Fig. 6C and D). Concomitantly, the percentage of epithelium decreases significantly (42.5 ± 13.5% in *E. glaucinus* and 38.1 ± 13.4% in *M. molossus*) with an increase in the luminal and interstitial percentages (L: 32.4 ± 6.5%; It: 25.1 ± 13.4% in *E. glaucinus* and L: 24.1 ± 6.0%; It: 47.9 ± 11.9% in *M. molossus*) (Fig. 6A and B). The percentage of cells in the cauda epididymis is not statistically different from the corpus in either species. On the other hand, the tubular and luminal diameters and percentage of epithelium, lumen and interstitial tissue differ significantly between the two species. The quantity of spermatozoa in the lumen increases greatly.

DISCUSSION

Numerous morphological and biochemical studies have been carried out on the epididymis of different experimental and pet animals, showing that its morphological structure is partially conserved in the majority of mammals (Alsum and Hunter 1978; Tingari and Moniem 1979; Oke et al. 1988; Schimming et al. 1997; Axnér et al. 1999; Aguilera-Merlo et al. 2005; Domeniconi et al. 2007; Takano 2007; Ahmed et al. 2009). These studies elucidate from the structural characteristics (Schimming and Vicentini 2001;

Schimming et al. 2002), their participation in the maturation of spermatozoa (Hinton 1990; Amann et al. 1993) to the morphological changes during aging (Haidl et al. 1996; Serre and Robaire 1998).

Studies in wild animals, such as bats, which are subject to seasonal and abiotic changes (temperature, rainfall and availability of food) are still scarce (Aguilera-Merlo et al. 2005). Compared to females, male reproductive system of bats has been poorly investigated. Most interpretations of their reproductive status are based on assessments of changes externally visible in the size of the testis and epididymis and sperm storage and longevity in cauda epididymis in species of temperate area (Racey 1973; Krutzsch et al. 1982, 2002; Crichton et al. 1994; Krutzsch 2000; Encarnação et al. 2004; León-Galván et al. 2005; Cervantes et al. 2008).

By the morphological constitution and functional complexity of the epididymis, it has been divided into three to five segments which are reported to be species-specific (Goyal 1985; Schimming et al. 1997). Three segments are always recognized in most species: the caput, corpus, and cauda epididymis. In the species of bats analyzed, despite belonging to different genera and differ morphologically, the epididymis showed similar morphological characteristics, in which four segments were easily recognized, the initial, caput, corpus and cauda segments.

The initial segment was not recognized or reported in previous studies involving other species of bats (Krutzsch 2000). In the species analyzed, the initial segment is small, and it is located between the tubules of the rete testis and the epididymal caput, which is characterized by a greater epithelial height and absence of spermatozoa in the lumen. These features suggest a fast transit of spermatozoa in this segment, as has been observed in hamster, cat and dog (Vicentini and Orsi 1987; Viotto et al. 1988; Schimming et al. 1997).

Both species analyzed showed a pseudostratified columnar epithelium with extensive stereocilia, composed of three main cell types: apical, basal and principal cells. Other cell types, clear and halo cells, were also observed, but in smaller numbers and in only some segments. These cell types were also observed in other mammals (Calvo et al. 1999; Aguilera-Merlo et al. 2005), and similarly, the presence, abundance and localization of each cell type varied between the species.

As in the majority of mammals, the principal cell was the predominant cell type in all segments, and their abundance varied little between the segments. In the rodent *Lagostomus m. maximus* (viscacha), Aguilera-Merlo et al. (2005) observed a variation in percentage between 40-60% over the segments, while in our study the values were higher, between 77-85%. On the other hand, these authors observed a high percentage of narrow-clear (3-9%) and halo cells (10-20%), which were observed in smaller proportions and only sporadically in the bats analyzed here.

In the two species of bats analyzed, the apical cells were observed in all epididymal segments, and not only in the initial segment and caput epididymis, as observed in gerbil epididymis (Domeniconi et al. 2007). Furthermore, the basal cells were found to be isolated cells that do not form continuous layers, unlike that observed in the dog (Schimming et al. 1997).

The observation of giant vacuoles in the principal cells in the initial segment in *M. molossus* was also reported for viscacha by Aguilera-Merlo et al. (2005) who noted the formation of these vacuoles during the period of gonadal regression. In the present study, the animals were not undergoing gonadal regression in order that the testes were functionally active and spermatozoa were observed in corpus and cauda epididymis segments. Fabián and Marques (1989) reported for *M. molossus*, the occurrence of pregnant females in November, March and April, and males with sperm in the epididymis in March, April, June, July, October and November.

The results of this study in association with those observed by Fabián and Marques (1989) confirm the polyoestry condition for *M. molossus* and *E. glaucinus*, supporting studies that have established that the species of tropical bats are primarily polyestrous with variation in the number of breeding cycles undergone annually (Krutzsch 2000). Moreover, it may well be that at any one time throughout the year not all males in the population are reproductively active at the same time.

In comparing the two species, we noted that the tubular and luminal diameters and percentage of interstitial tissue differed significantly in all segments. The epithelial height in both is greater in the initial segment and decreases until the cauda epididymis. In relation to the luminal diameter, both species showed a progressive increase from the initial segment to the cauda epididymis. The percentage of epithelium, lumen and interstitial tissue varied between the two species, sometimes with *M. molossus* showing a significantly higher percentage and at other times, *E. glaucinus*. In both species, the principal cell was the most abundant (> 77%), followed by basal cells at approximately 13% and apical cells at 4% in all segments.

In summary, ours results show that although the species analyzed belong to different genera and have different body sizes and different breeding cycles, the epididymis of both exhibits some similarities and interesting differences in morphometry and composition compared to the majority of mammals. Its basic morphological characterization is important for future studies, such as comparison with other species of bats that show different epididymal morphology and prolonged sperm storage (e.g., vespertilionid species), investigation of its ultrastructure, elucidation of its function in sperm maturation and storage, and the influence of androgens (testosterone) on its seasonal changes.

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